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Evaluation of Elastase and α_1-Proteinase Inhibitor-Elastase Uptake by Polymorphonuclear Leukocytes and Evidence of an Elastase-Specific Receptor

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Summary: Neither resting nor stimulated isolated human polymorphonuclear leukocytes did bind or ingest preformed complexes of α_1-proteinase inhibitor and unlabeled/^{125}I-labeled human leukocyte elastase. In contrast, granulocytes bound unlabeled/^{125}I-labeled elastase and the extent of binding was reduced in the presence of respiratory burst stimulators, such as 4β-phorbol 12β-myristate 13α-acetate, *E. coli* endotoxin, and N-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine. In association/dissociation and competition inhibition experiments it was demonstrated that granulocyte-elastase binding was specific and saturable. From *Scatchard* and non-linear regression analysis there was evidence of a two-class receptor model with independent binding sites. Calculated by the non-linear regression method assuming a two-class receptor model the characteristics of the high affinity/low capacity binding site were $K_1 = 216 \pm 129 \cdot 10^6\ l \cdot mol^{-1}$ ($\bar{x} \pm s$; $n = 3$) and $R_1 = 1.38 \pm 0.95$ nmol · l^{-1} corresponding to $0.083 \cdot 10^6$ receptors per cell, whereas the low affinity/ high capacity binding site had the characteristics $K_2 = 0.50 \pm 0.09 \cdot 10^6\ l \cdot mol^{-1}$ and $R_2 = 237 \pm 103$ nmol · l^{-1} corresponding to $14.3 \pm 6.2 \cdot 10^6$ receptors per cell.

Untersuchungen zur Elastase- und α_1-Proteinaseinhibitor-Elastase-Aufnahme polymorphkerniger Leukocyten und Nachweis eines Elastaserezeptor-Systems

Zusammenfassung: Komplexe aus α_1-Proteinaseinhibitor und unmarkierter/markierter humaner Leukocyten-Elastase werden von isolierten menschlichen polymorphkernigen Leukocyten weder unter Ruhe- noch unter Stimulations-Bedingungen aufgenommen. Dagegen binden Granulocyten Elastase/[^{125}I]Elastase, wobei die [^{125}I]Elastase-Bindung in Gegenwart der respiratory burst-Stimulantien 4β-Phorbol-12β-myristat-13α-acetat, *E. coli*-Endotoxin und Formyl-methionyl-leucyl-phenylalanin erniedrigt ist. Aus Assoziations-/Dissoziations- und Kompetitions-Inhibitions-Experimenten geht hervor, daß die Granulocyten-Elastase-Bindung spezifisch und sättigbar ist. *Scatchard-* und nichtlineare Regressions-Analyse deuten auf ein zweiklassiges Modell unabhängiger Rezeptoren hin. Aus der nichtlinearen Regressionsanalyse ergeben sich unter Annahme eines zweiklassigen Rezeptormodells die Bindungskonstanten $K_1 = 216 \pm 129 \cdot 10^6\ l \cdot mol^{-1}$ ($\bar{x} \pm s$; $n = 3$) und $R_1 = 1{,}38 \pm 0{,}95$ nmol · l^{-1} entsprechend $0{,}083 \cdot 10^6$ Rezeptoren pro Zelle für die hochaffine/niedrigkapazitive Bindungsstelle, während die niedrigaffine/hochkapazitive Bindungsstelle die Konstanten $K_2 = 0{,}50 \pm 0{,}09 \cdot 10^6\ l \cdot mol^{-1}$ und $R_2 = 237 \pm 103$ nmol · l^{-1} entsprechend $14{,}3 \pm 6{,}2 \cdot 10^6$ Rezeptoren pro Granulocyt besitzt.

Introduction

As with other cells of the mononuclear phagocytic system human alveolar macrophages are able to endocytose proteins and protein complexes by membrane receptor mediated processes. Thus, human neutrophil leukocyte elastase as well as α_2-macroglobulin-elastase complexes are pinocytosed and phagocytosed, respectively, by alveolar macrophages, suggesting that at least some of the elastase activity found in macrophages may be ingested rather than formed de novo (1—4). A previous report from this laboratory presented evidence that bronchoalveolar lavage-derived human granulocytes accumulate appreciable amounts of α_1-proteinase inhibitor-elastase complexes during passage across the blood-air barrier (5). In compartments such as the interstitial space where α_2-macroglobulin is likely to be present only in low concentrations (1) elastase clearance must be supposed to occur predominantly via complex formation with α_1-proteinase inhibitor and elimination by the reticuloendothelial system cells.

The present work was undertaken to clarify whether granulocytes could provide an additional pathway for uptake and elimination of elastase and/or α_1-proteinase inhibitor-elastase complexes, thus giving this cell a protective role in particular pathophysiological situations additionally to its known dangerous release reactions during stimulation (6—8). Uptake of [^{125}I]elastase/elastase and α_1-proteinase inhibitor-[^{125}]elastase/elastase complexes by isolated human polymorphonuclear leukocytes was investigated under non-stimulation conditions and during respiratory burst stimulation with soluble and particulate substances. α_1-Proteinase inhibitor-elastase complex ingestion was estimated by nephelometrically measured extracellular decrease of the complex concentration as well as cellular radioactivity increase by employment of [^{125}I]labeled elastase. In vitro binding of elastase to human granulocytes for association and dissociation experiments as well as for receptor studies was determined by the use of [^{125}I]elastase.

Materials and Methods

Reagents and equipment

Fresenius, Bad Homburg, FRG: sodium citrate solution (31.3 g/l)

Pharmacia Fine Chemicals, Uppsala, Sweden: Percoll for density gradient centrifugation; columns PD-10 Sephadex G-25 M

Boehringer, Mannheim, FRG: phosphate buffered saline Dulbecco; Minimal Essential Medium Dulbecco for chemiluminescence without Phenol Red, without glutamine

E. Merck, Darmstadt, FRG: *Türk*'s solution (acetic acid gentian violet solution) for leukocyte counting; sodium chloride; Triton X-100; dimethylsulphoxide; monosodium phosphate · H_2O; disodium phosphate · 2 H_2O; sodium iodide; tris-(hydroxymethyl)aminomethane; N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid

Sigma Chemical Co. St. Louis, MO, U.S.A.: α_1-antitrypsin, from human plasma, partially purified; albumin, bovine fraction V, 96—99%; zymosan A from *S. cerevisiae* yeast; chloramine T, trihydrate, crystalline; sodium bisulphite (sodium metabisulphite), grade I; 4β-phorbol 12β-myristate 13α-acetate; N-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; lipopolysaccharide from *E. coli* serotype No. 055: B5, phenol extract

Bachem, Bubendorf, Switzerland: methoxysuccinyl-*L*-Ala-*L*-Ala-*L*-Pro-*L*-Val-*p*-nitroanilide

Blood bank, Medizinische Hochschule Hannover, FRG: venous blood from male blood donors anticoagulated with 31.3 g/l sodium citrate solution (9 vol of blood + 1 vol of citrate solution); AB plasma, normal plasma from a AB/Rh pos blood donor

Immuno Diagnostika GmbH, Heidelberg, FRG: anti-human-α_1-antitrypsin from goat; immunoneph Reference Standard Human Proteins; immunoneph Norm Control Human Proteins; Immuno-Video-Nephelometer; polyethylene glycol buffer concentrate, 400 g/l

Amersham Buchler GmbH + Co KG, Braunschweig, FRG: Iodine-125, iodide, carrier-free, 100 Ci/l, 3.7 TBq/l

Laboratorium Prof. Dr. Berthold, Wildbad, FRG: multi-crystal gamma counter LB 2100

Isolation of human polymorphonuclear leukocytes

Polymorphonuclear leukocytes were isolated from venous blood of donors anticoagulated by the addition of 31.3 g/l sodium citrate solution (9 vol of blood + 1 vol of citrate solution). In a slightly modified *Hjorth*'s procedure (9) 4 ml were layered on a two-step discontinuous Percoll gradient (densities of 1.077 and 1.095 g/cm^3) in a 13 ml polystyrene tube and centrifuged at 400 *g* at 22 °C for 20 min. Granulocytes were harvested, washed twice with phosphate buffered saline by centrifugation (600 *g* for 10 min at 22 °C), resuspended with 200—500 µl of minimal essential medium buffer solution and counted using a *Neubauer* haemocytometer after cell staining with *Türk*s solution.

α_1-Proteinase inhibitor-elastase complex formation

Elastase/α_1-proteinase inhibitor titration

A stock solution of human α_1-proteinase inhibitor (2 g/l saline solution, 0.154 mol/l) was diluted with minimal essential medium buffer solution containing bovine serum albumin, 1 g/l, to give concentrations of 3.125; 6.25; 12.5; 25.0 and 50.0 mg/l α_1-proteinase inhibitor. From each of these solutions, an aliquot of 150 µl was mixed with 150 µl of elastase solution[1]) (3.72 mg/l elastase in minimal essential medium buffer solution with bovine serum albumin, 1 g/l) and incubated for 30 min at 22 °C. Non-complexed elastase was determined enzymatically as follows: 800 µl of buffer solution (0.1 mol/l N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid, 2 mol/l sodium chloride, 0.5 g/l Triton X-100, pH 8.0) were mixed with 100 µl of sample (above reaction mixtures) and 100 µl of methoxysuccinyl-*L*-Ala-*L*-Ala-*L*-Pro-*L*-Val-*p*-nitroanilide solution (2 g/l dimethylsulphoxide) and the absorbance was recorded at 405 nm and 25 °C. 1 unit of elastase activity was defined as the substrate turnover of 1 µmol/min under the test conditions employed.

[1]) Human leukocyte elastase (9.3 g/l elastase, charge XXIII, in 0.02 mol/l acetic acid/0.15 mol/l sodium chloride, pH 5.5, frozen at −70 °C) was a gift from Dr. *S. Neumann*, E. Merck, Darmstadt, FRG.

α_1-Proteinase inhibitor-elastase (1 : 1) complex formation

300 µl of α_1-proteinase inhibitor solution (283.2 mg/l minimal essential medium) were incubated with 300 µl of elastase solution (36.8 mg/l) for 30 min at 22 °C, then diluted 1 : 8 with minimal essential medium buffer solution with bovine serum albumin, 1 g/l, to give a 20 mg/l solution of the formed α_1-proteinase inhibitor-elastase complex.

Opsonisation of zymosan

1 g of zymosan A was incubated with 20 ml of AB plasma at 37 °C for 30 min, centrifuged (800 *g* at 22 °C for 10 min), washed twice with phosphate-buffered saline, once with minimal essential medium buffer solution, resuspended in minimal essential medium buffer, 50 g/l, and frozen in aliquots at −70 °C.

Phagocytosis experiments with α_1-proteinase inhibitor-elastase complex

Phagocytosis experiments were done with a granulocyte suspension ($14.7 \cdot 10^9$/l minimal essential medium buffer solution with bovine serum albumin, 1 g/l), α_1-proteinase inhibitor-elastase solution (20 mg/l), suspension of opsonized zymosan (50 g/l) and minimal essential medium buffer solution with bovine serum albumin, 1 g/l, according to the following scheme: (volumes in microliters)

	incubation mixtures		
	1	2	3
granulocyte suspension	120	120	—
α_1-proteinase inhibitor-elastase solution	120	120	120
zymosan suspension	12	—	—
minimal essential medium with bovine serum albumin, 1 g/l	—	12	132

After incubation at 37 °C for 60 min the reaction mixtures were centrifuged (12 000 *g* at 22 °C for 4 min) and the supernatants were analysed nephelometrically for α_1-proteinase inhibitor-elastase concentrations.

Nephelometrical determination of α_1-proteinase inhibitor-elastase

α_1-Proteinase inhibitor-elastase complex concentrations were determined by employment of the reagent sets, protocols and instrumentation of Immuno Diagnostika GmbH for the determination of α_1-proteinase inhibitor.

Iodination of human leukocyte elastase and enzyme activity

A modification of the chloramine T method of *Greenwood & Hunter* (10) and *Fink & Güttel* (11) was used to iodinate human leukocyte elastase. All reagents were dissolved in 0.05 mol/l sodium phosphate buffer solution, pH 7.5. To 20 µl of diluted $Na^{125}I$ solution (6.7 µCi, 247.9 KBq) 5 µl of elastase solution (2 g/l) and 5 µl of chloramine T solution (5 g/l) were added and mixed. After 60 s at 22 °C, 50 µl of sodium bisulphite solution (1.2 g/l) and 200 µl of sodium iodide solution (4.8 g/l in phosphate buffer containing bovine serum albumin, 10 g/l) were added, the reaction mixture was applied to a column PD-10 (Pharmacia) and [^{125}I]elastase was eluted with buffer solution (0.1 mol/l tris-(hydroxymethyl) aminomethane hydrochloride, 0.5 mol/l sodium chloride, bovine serum albumin, 10 g/l, pH 7.5) in 500 µl fractions with an elution rate of about 1 ml/min. Fractions 8—10 showed peak radioactivity corresponding to the elution volume of elastase. They were pooled to give a concentration of 7.5 mg/l [^{125}I]elastase; the specific radioactivity was 0.24 Ci/g, or 8.88 GBq/g. The biological activity of [^{125}I]elastase was tested by titration with α_1-proteinase inhibitor as described above.

Phagocytosis experiments with α_1-proteinase inhibitor-[^{125}I]elastase complex

α_1-Proteinase inhibitor-[^{125}I]elastase complex was formed by incubation of 480 µl of [^{125}I]elastase solution (7.5 mg/l) and 240 µl of α_1-proteinase inhibitor solution (139 mg/l) for 20 min at 22 °C. Using this solution, phagocytosis experiments were performed as follows: (volumes in microliters)

	incubation mixtures			
	1	2	3	4
granulocyte suspension ($20.6 \cdot 10^9$/l)	150	150	—	—
opsonized zymosan (50 g/l)	10	—	10	—
α_1-proteinase inhibitor-[^{125}I]elastase solution (51.3 mg/l)	150	150	150	150
minimal essential medium with bovine serum albumin, 1 g/l	—	10	150	160

After 30 min at 37 °C the tubes were centrifuged at 12 000 *g* for 1 min at 22 °C, the supernatants were separated as quantitatively as possible, the remaining cell pellets were washed four times with each 500 µl of minimal essential medium (with bovine serum albumin, 1 g/l), then the cell pellets were resuspended in minimal essential medium with bovine serum albumin, 1 g/l, and transferred into a second tube by rinsing the original tube another two times each with 200 µl of minimal essential medium with bovine serum albumin, 1 g/l (to determine radioactivity adsorption in the original tube) and centrifuged; the supernatants were discarded, 500 µl of water were added (to lyse the cells for the determination of incorporated radioactivity), centrifuged and the supernatants separated from the sediments. All supernatants, cell pellets and sediments were analysed for radioactivity by a multi-channel gamma counter.

Granulocyte-[^{125}I]elastase binding

Binding of [^{125}I]elastase by stimulated and non-stimulated granulocytes was investigated according to the following scheme: (volumes in microliters)

	incubation mixtures					
	1	2	3	4	5	6
granulocyte suspension ($13.6 \cdot 10^9$/l)	150	150	—	150	150	150
opsonized zymosan (12.5 g/l)	50	—	50	—	—	—
4β-phorbol 12β-myristate 13α-acetate (35 µmol/l)	—	—	—	50	—	—
N-formyl-methionyl-leucyl-phenylalanine (100 µmol/l)	—	—	—	—	50	—
endotoxin (413 mg/l)	—	—	—	—	—	50
minimal essential medium with bovine serum albumin, 2 g/l	—	50	150	—	—	—
[^{125}I]elastase (4.8 mg/l)	150	150	150	150	150	150

After incubation at 37 °C for 30 min with gentle agitation, the reaction mixtures were centrifuged (2000 *g*, 22 °C, 5 min), a 300 µl aliquot of the supernatant was removed, 600 µl of minimal essential medium (with bovine serum albumin, 2 g/l) were added to the cell pellet, resuspended by vortexing and centrifuged under the above conditions. This washing procedure was repeated three times. Then the cell pellet was resuspended with 600 µl of minimal essential medium (with bovine serum albumin, 2 g/l) and transfered into a second tube by rinsing the original tube another two times each with 200 µl of minimal essential medium with bovine serum albumin, 2 g/l (to determine the adsorption of [^{125}I]elastase on to the tube surface). After sedimentation of the cells by centrifugation and removing the supernatant, 800 µl of water were added (to lyse the cells for the determination of incorporated radioactivity), centrifuged and the supernatant was separated from the sediment. All supernatants, cell pellets and sediments were analysed for radioactivity.

Association and dissociation of [^{125}I]elastase and granulocytes

Association of [^{125}I]elastase (9.29 Ci/g, 344 GBq/g, from another iodination procedure) and isolated human polymorphonuclear leukocytes was investigated as follows: (volumes in microliters)

	incubation mixtures		
	1	2	3
granulocyte suspension ($13.3 \cdot 10^9$/l)	1100	1100	1100
minimal essential medium with bovine serum albumin, 2 g/l	90	65	65
[^{125}I]elastase solution (24 mg/l)	10	10	10
elastase solution 1 (930 mg/l)	—	25	—
elastase solution 2 (9300 mg/l)		—	25

Incubation was performed at 24 °C with gentle agitation. At times 0, 5, 10, 20 and 30 min, 200 µl were transferred into 13 ml polystyrene tubes and total radioactivity was measured for 1 min. After subsequent addition of 10 ml of prechilled (4 °C) phosphate-buffered saline solution (with bovine serum albumin, 2 g/l) the tubes were centrifuged at 2000 *g* for 2 min, the supernatants were removed totally by suction, the radioactivity of the cell pellets was measured and the fraction of [^{125}I]elastase bound to granulocytes was calculated. Dissociation experiments were performed as follows: after a preceeding association experiment by incubation of 2 ml of granulocyte suspension ($10 \cdot 10^9$/l) and 80 µl of [^{125}I]elastase (24 mg/l) at 24 °C for 30 min, [^{125}I]-elastase binding was determined by dilution of 100 µl of the incubation mixture with 10 ml of prechilled (4 °C) phosphate-buffered saline solution (with bovine serum albumin, 2 g/l), centrifugation at 2000 *g* for 2 min, removal of the supernatant and radioactivity counting of the cell pellet. The radioactivity of 100 µl of the incubation mixture served as the fraction 1.0 reference value. Then the incubation mixture was centrifuged at 2000 *g* for 5 min, the supernatant was removed and the cell pellet was rinsed with the original volume of phosphate-buffered saline solution (with bovine serum albumin, 2 g/l) at 4 °C. After centrifugation at 2000 *g* for 1 min the supernatant was removed and the cell pellet was resuspended with the original volume of ice-cold phosphate-buffered saline solution (with bovine serum albumin, 2 g/l). Each 500 µl of this suspension were diluted

(i) with 50 ml of phosphate-buffered saline solution (with bovine serum albumin, 2 g/l) and

(ii) with 50 ml of phosphate-buffered saline solution (with bovine serum albumin, 2 g/l) and 30 µl of elastase solution (9.3 g/l) and kept at 24 °C with gentle agitation.

After 0, 5, 10, 20, 30, 60, and 120 min, 5 ml of the suspension were centrifuged at 2000 *g* for 2 min. Several 1 ml aliquots of the supernatant were analysed for radioactivity and their mean was referred to the mean of a replicate measurement of several aliquots of (i) and (ii), respectively, without centrifugation, as fraction 1.0 reference values.

Calculation of the [^{125}I]elastase dissociation was performed according to the formula:

$$\text{fraction of dissociation} = \frac{\text{counts/min}_{\text{supernatant at t min}}}{\text{counts/min}_{\text{total}} - \text{counts/min}_{\text{supernatant at 0 min}}} - \frac{\text{counts/min}_{\text{supernatant at 0 min}}}{\text{counts/min}_{\text{total}} - \text{counts/min}_{\text{supernatant at 0 min}}}$$

fraction of [^{125}I]elastase binding = 1.0 − fraction of dissociation.

Competition inhibition experiments

Receptor characteristics were evaluated according to the following scheme:

Before pipetting any reagent all tubes were rinsed with 10 ml of phosphate-buffered saline (with bovine serum albumin, 2 g/l), which was carefully removed by suction (to avoid adsorption of elastase onto the vessel wall). All experiments were done in duplicate in conical 12 ml polystyrene centrifugation tubes.

1. elastase solution (0.03—312.6 µmol/l) in minimal essential medium with bovine serum albumin, 2 g/l — 40 µl
2. [^{125}I]elastase solution (37.5 nmol/l and 9.3 Ci/g, 344.1 GBq/g) — 40 µl

total radioactivity counting

3. granulocyte suspension (7.1; 11.2; $15.5 \cdot 10^9$/l) in minimal essential medium with bovine serum albumin, 2 g/l — 300 µl

After incubation at 24 °C for 40 min with agitation 10 ml of ice-cold phosphate-buffered saline solution (with bovine serum albumin, 2 g/l) were added and the tubes were centrifuged at 2000 *g* for 2 min. For radioactivity counting of the supernatant one 500 µl aliquot was withdrawn, then the supernatant was removed by suction except for a remaining volume of 100 µl including the cell pellet, which was also counted. Calculation of the fraction of [^{125}I]elastase binding was performed according to the formula:

$$\text{fraction of binding} = \frac{\text{counts/min}_{\text{sed}} - 0.2 \cdot \text{counts/min}_{\text{sup}}}{\text{counts/min}_{\text{total}}}$$

$\text{Counts/min}_{\text{sed}}$ = radioactivity of the cell pellet including 100 µl of the supernatant
$\text{counts/min}_{\text{sup}}$ = radioactivity of 500 µl of the supernatant
$\text{counts/min}_{\text{total}}$ = total radioactivity of the reaction mixture
The fraction of binding was normalized for $10 \cdot 10^9$/l granulocytes in the incubation mixture.

Calculation of receptor characteristics

In a first approach elastase receptor affinity and concentration were calculated by employment of the *Scatchard* plot (12). Specific binding was calculated from total binding values by subtraction of the non-specific binding determined in the presence of the highest concentration of unlabeled elastase. Then B/F ratios were calculated and plotted versus the corresponding

concentrations of bound elastase. The receptor system could be described by two straight lines of different slopes ($K_{high\ affinity}$ and $K_{low\ affinity}$) and abscissa intercept points ($R_{low\ capacity}$ and $R_{high\ capacity}$).

The binding sites per cell were calculated by:

$$\text{binding sites per cell} = \frac{6.023 \cdot 10^{23} \cdot \text{mol/l}_{bound}}{\text{cell count/l}}$$

In a second approach the binding parameters were extracted by iteration using a non-linear regression analysis for a one-class as well as for a two-class receptor model according to l. c. (13), whereby iteration was started with constants determined by the *Scatchard* analysis.

Results

α_1-Proteinase inhibitor-elastase complex formation

Equilibrium titration of elastase with α_1-proteinase inhibitor resulted in a α_1-proteinase inhibitor: elastase ratio of 7.7 : 1 for total inhibition of elastase activity (fig. 1).

Phagocytosis experiments with α_1-proteinase inhibitor-elastase complex

The results of the phagocytosis experiments show that there was no uptake by polymorphonuclear leukocytes of the preformed α_1-proteinase inhibitor-elastase complex, either by resting or by zymosan-stimulated granulocytes (tab. 1).

Tab. 1. Phagocytosis experiments (37 °C, 60 min) with α_1-proteinase inhibitor-elastase complex (9.52 mg/l ≙ 6.0 mg/l α_1-proteinase inhibitor) and granulocytes ($7 \cdot 10^9$/l) in the absence and presence (2.38 g/l) of opsonized zymosan (+ = with; Ø = without).

	Nephelometrically determined α_1-proteinase inhibitor concentration of supernatants (mg/l; n = 4)		Fraction of granulocyte 'uptake'
	$\bar{x}$	SEM	
Incubation 1 (+ granulocytes + zymosan)	5.9	0.25	+0.017
Incubation 2 (+ granulocytes Ø zymosan)	6.3	0.18	−0.05
Incubation 3 (Ø granulocytes Ø zymosan)	5.7	0.20	+0.05

Iodination of elastase and the enzymatic activity of [^{125}I]elastase

The determination of the α_1-proteinase inhibitor/[^{125}I]elastase inhibition interrelationship demonstrated a slight increase of the biological activity caused by iodination in comparison to the non-iodinated compound; the α_1-proteinase inhibitor/elastase ratio for complete inhibition under equilibrium conditions increased from 7.7 (fig. 1) to 9.3 after iodination (fig. 2).

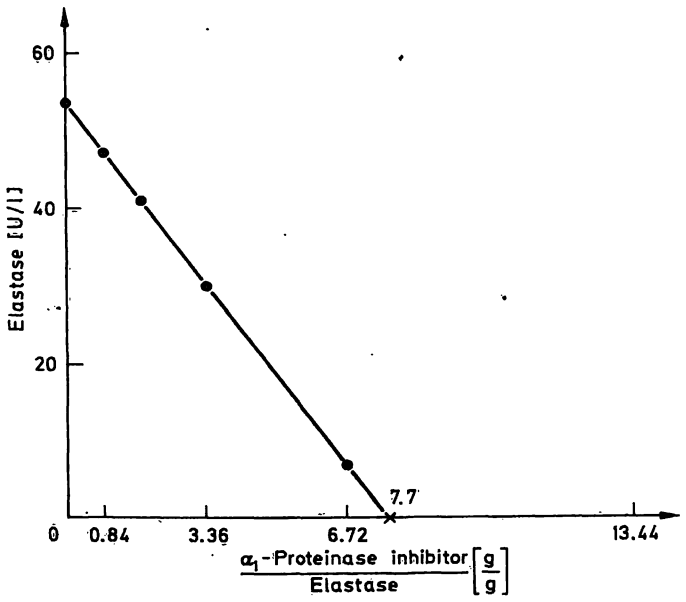

Fig. 1. Human leukocyte elastase enzyme activity in dependence on the α_1-proteinase inhibitor/elastase ratio determined under equilibrium conditions.

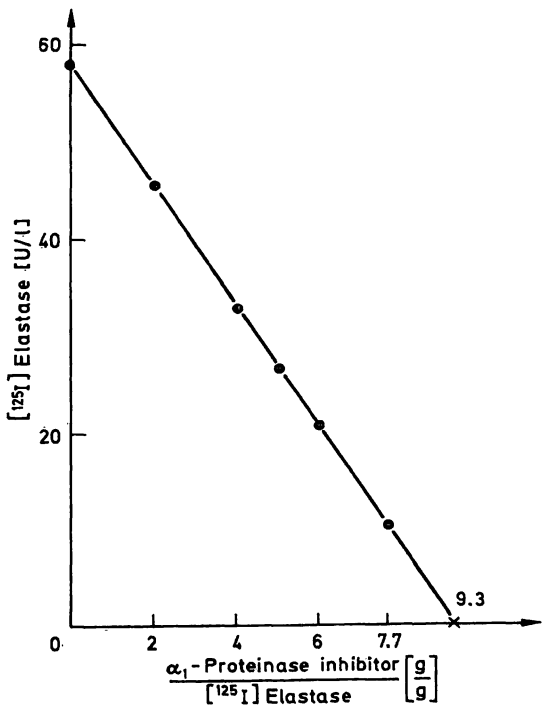

Fig. 2. [^{125}I]Elastase enzyme activity in dependence on the α_1-proteinase inhibitor/[^{125}I]elastase ratio determined under equilibrium conditions.

Phagocytosis experiments with α_1-proteinase inhibitor-[^{125}I]elastase complex

By employment of the radiolabeled α_1-proteinase inhibitor-[^{125}I]elastase complex, cellular uptake and non-specific adsorption of the complex were investigated. The results are shown in figure 3. In comparison with the particle-bound radioactivity after the fourth washing step, it could be demonstrated that the presence of zymosan increased the particle-bound radioactivity (granulocytes + zymosan) to a fraction of about 0.03, whereas only 0.01 – 0.015 were found in the absence of zymosan; furthermore, most of this portion resulted from radioactivity adsorption onto the vessel wall, as shown by the data for T, L, and A in figure 3. From the experiments with granulocytes and α_1-proteinase inhibitor-[^{125}I]elastase complex, it was calculated that less than 0.003 of the radioactivity fraction was bound by granulocytes, indicating the absence of α_1-proteinase inhibitor-[^{125}I]elastase complex phagocytosis and/or cellular adsorption of this complex.

Binding of [^{125}I]elastase by polymorphonuclear leukocytes

Figure 4 shows [^{125}I]elastase binding by isolated granulocytes under stimulating and non-stimulating conditions. In the presence of the soluble stimuli, 4β-phorbol 12β-myristate 13α-acetate, *E. coli* endotoxin and N-formyl-methionyl-leucyl-phenylalanine, cellular binding ranged from 0.07 to 0.13 after the fourth washing step, whereas in the absence of any stimulus the binding was found to be 0.145. The data observed after further cell treatment (procedures T and L) as well as the low adsorption values (fractions of 0.0039 – 0.0074) indicated that [^{125}I]elastase was highly bound by granulocytes, presumably in a specific manner. From binding experiments with zymosan, an extremely high [^{125}I]elastase binding to zymosan was seen, which was rarely altered in the presence of granulocytes, thereby indicating that strong adsorptive forces of zymosan and elastase surpass any influence of granulocytes.

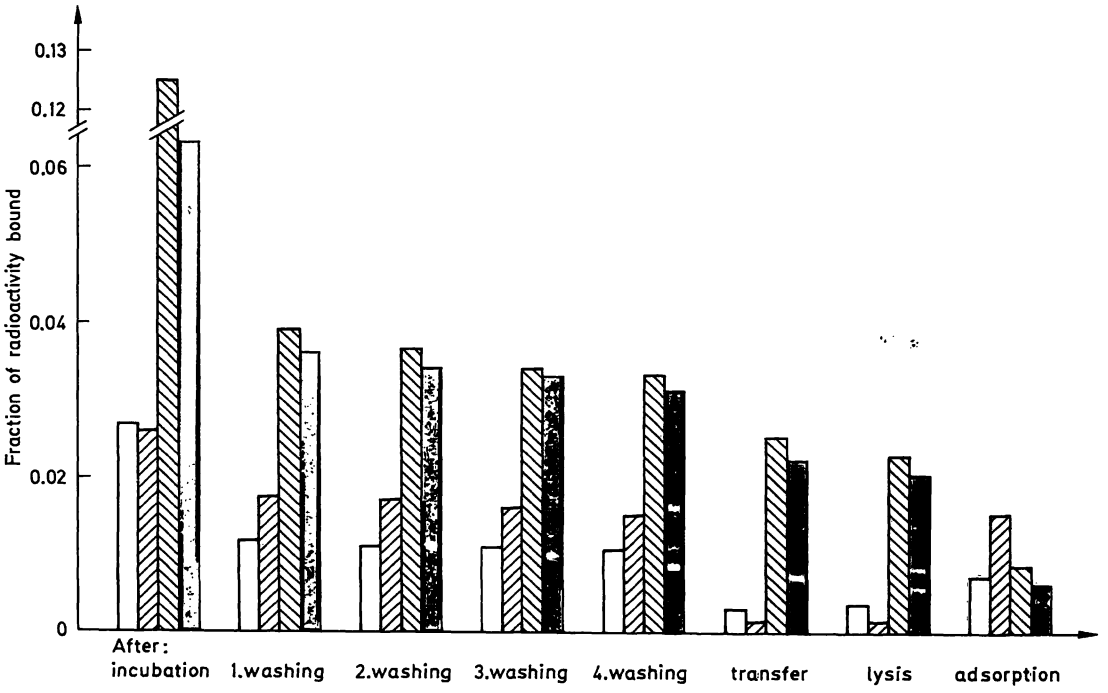

Fig. 3. Incubation of granulocytes with α_1-proteinase inhibitor/[^{125}I]elastase complex in the absence and presence of opsonized zymosan.
The columns represent
data after incubation
1. washing step
2. washing step
3. washing step
4. washing step
transfer into a second tube
water lysis in the second tube and
remaining radioactivity of the original tube (adsorption).

	Granulocytes	Zymosan	α_1-Proteinase inhibitor/[^{125}I]elastase
■	+	+	+
▨	–	+	+
□	+	–	+
▨	–	–	+

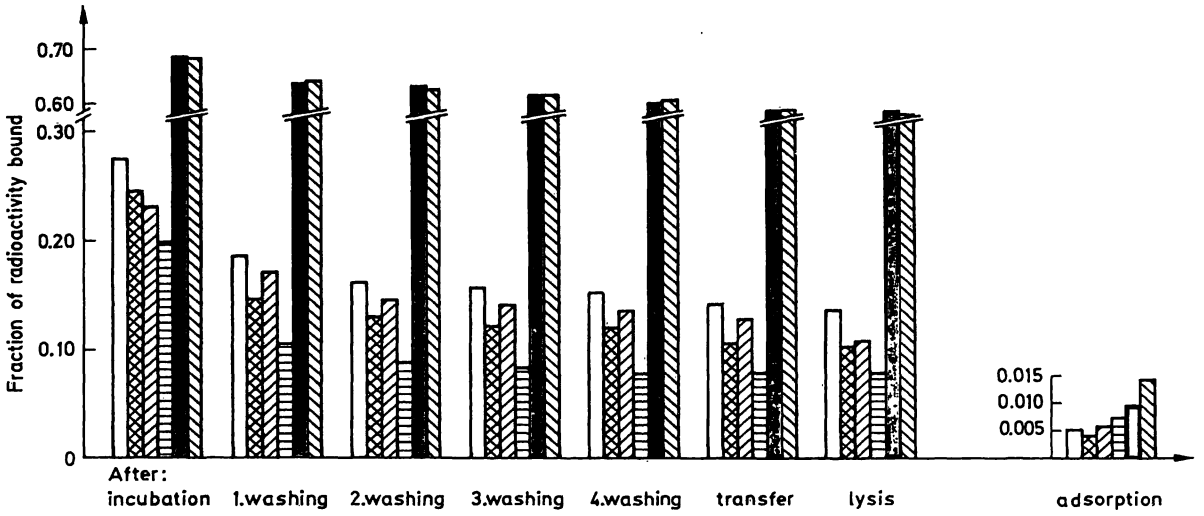

Fig. 4. Binding of [^{125}I]elastase (2.06 mg/l) to granulocytes ($5.8 \cdot 10^9$/l) in the absence and presence of zymosan (1.79 g/l), 4β-phorbol 12β-myristate 13α-acetate (5 μmol/l), N-formyl-methionyl-leucyl-phenylalanine (14.3 μmol/l), and *E. coli* endotoxin (59 mg/l).

The columns represent
data after incubation
1. washing step
2. washing step
3. washing step
4. washing step
transfer into a second tube
water lysis in the second tube and
remaining radioactivity of the original tube (adsorption).

	Granulocytes	Stimulant	[^{125}I]Elastase
■	+	Zymosan	+
□	+	–	+
▨	–	Zymosan	+
▤	+	4β-phorbol 12β-myristate 13α-acetate	+
▨	+	N-formyl-methionyl-leucyl-phenylalanine	+
▩	+	*E. coli* endotoxin	+

Association and dissociation of [^{125}I]elastase and polymorphonuclear leukocytes

Association of [^{125}I]elastase and granulocytes without and with different amounts of unlabeled elastase gave typical association kinetics as shown in figure 5. After a preceeding association of granulocytes and [^{125}I]elastase, dissociation experiments were performed at 24 °C by 1 : 100 dilution of the granulocyte suspension and following incubation with and without addition of an excess of unlabeled elastase (fig. 6). By dilution alone only weak and slow dissociation could be observed, which was hardly enhanced in the presence of unlabeled elastase, thus providing strong evidence for elastase-specific receptor binding sites on the granulocyte surface, but without site-site interactions as in the case of negative cooperatively acting receptor sites (14).

Competition inhibition experiments

Results from competition inhibition experiments with isolated granulocytes of three blood donors on different days were obtained according to the calculation procedures described in section 'Materials and Methods'.

(i) Plotting B/F ratios versus b (concentration of bound [^{125}I]elastase/elastase in nmol/l) gave *Scatchard* plots of the same behaviour in that they could be divided into two strictly defined straight lines assuming two independent binding sites, one of high affinity (K_1), low capacity (R_1), and another of low affinity (K_2), high capacity (R_2) (fig. 7). Computer assisted estimation of the binding parameters K and R was performed according to *Schwarz* (15), taking into account the mutual influences of the two regression lines.

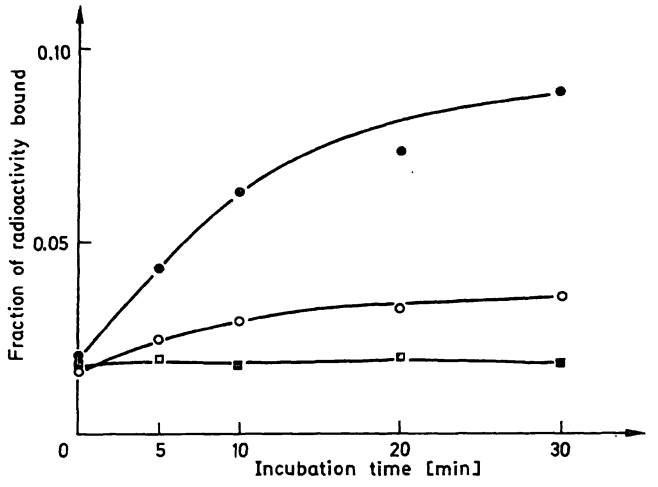

Fig. 5. Association kinetics of granulocytes ($12.2 \cdot 10^9$/l) and [^{125}I]elastase (0.2 mg/l) without and with unlabeled elastase (19.4 mg/l and 194 mg/l, respectively) at 24 °C.

[^{125}I]Elastase (mg/l)	Elastase (mg/l)
● 0.2	0
○ 0.2	19.4
■ 0.2	194.0

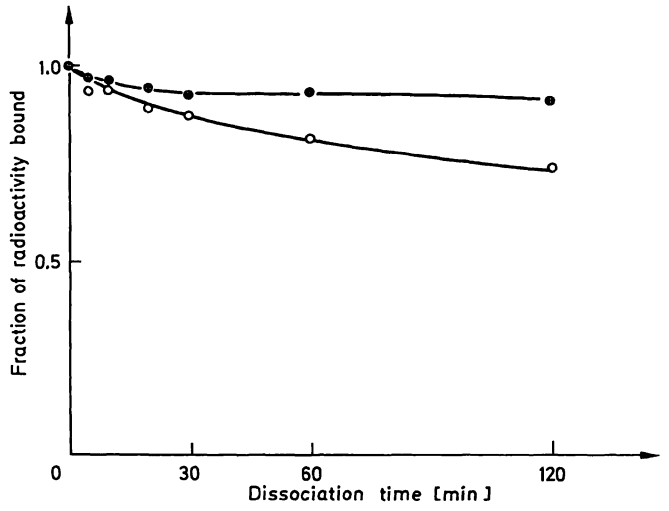

Fig. 6. Dissociation kinetics of [^{125}I]elastase from granulocytes at 24 °C by 1:100 dilution without and with an excess of unlabeled elastase (for experimental details see section 'Materials and Methods')

● 1:100 dilution
○ 1:100 dilution + unlabeled elastase (5.52 mg/l dissociation volume).

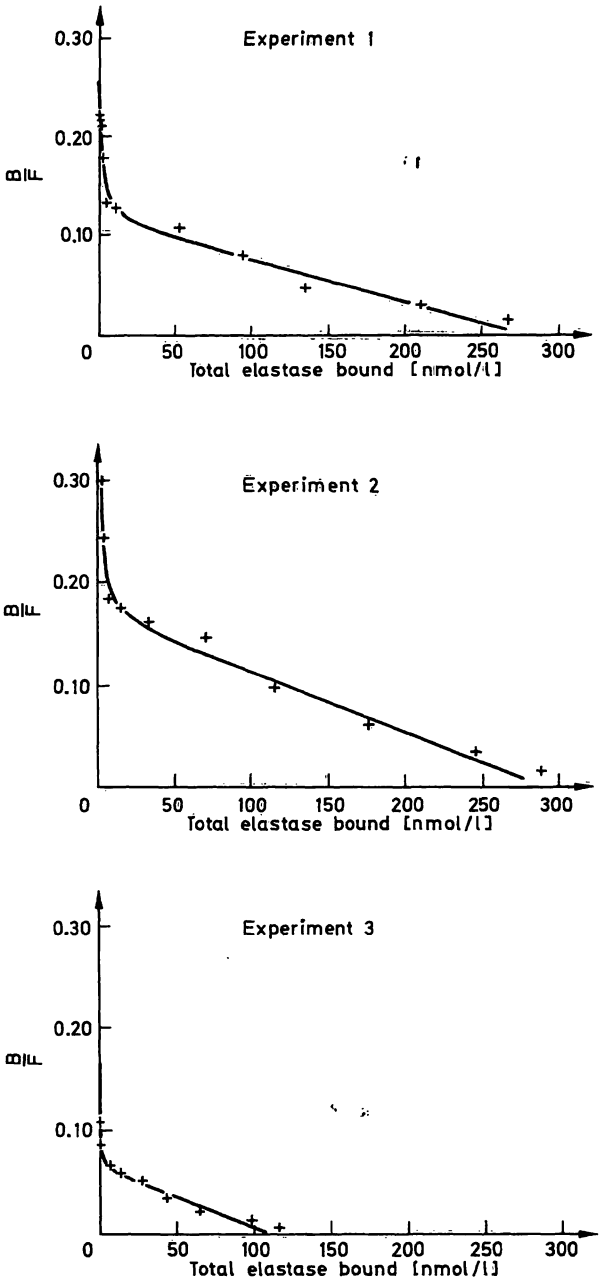

Fig. 7. *Scatchard* plots of three competition inhibition experiments with granulocytes and [^{125}I]elastase/elastase.

ordinate: B/F ratio
abscissa: concentration of [^{125}I]elastase/elastase bound in nmol/l.

(ii) By employment of a non-linear regression analysis (13) and by using the constants estimated from the *Scatchard* analysis as starting conditions for the iteration, the binding characteristics were extracted separately for a one-class as well as for a two-class binding model. Data are shown in table 2. The competition inhibition curves following iteration are given in figure 8.

Discussion

During migration of stimulated and phagocytosing polymorphonuclear leukocytes from the capillary vessel into the alveolar space they increase their content of α_1-proteinase inhibitor-elastase considerably (5). The aim of the present investigation was to determine whether α_1-proteinase inhibitor-elastase com-

Tab. 2. Binding parameters for the interaction of polymorphonuclear leukocytes and [^{125}I]elastase/elastase from non-linear regression analysis.

	K_1	R_1		K_2	R_2	
	(10^6 l · mol^{-1})	(nmol · l^{-1})	(receptors, 10^6 per cell)	(10^6 l · mol^{-1})	(nmol · l^{-1})	(receptors, 10^6 per cell)
Experiment 1						
1-class model	8.9	19.8	1.2			
2-class model	67.9	2.2	0.13	0.40	285	17.2
Experiment 2						
1-class model	4.8	52.3	3.2			
2-class model	302.3	1.6	0.1	0.56	307	18.5
Experiment 3						
1-class model	2.5	32.6	2.0			
2-class model	278.7	0.34	0.02	0.54	118	7.1

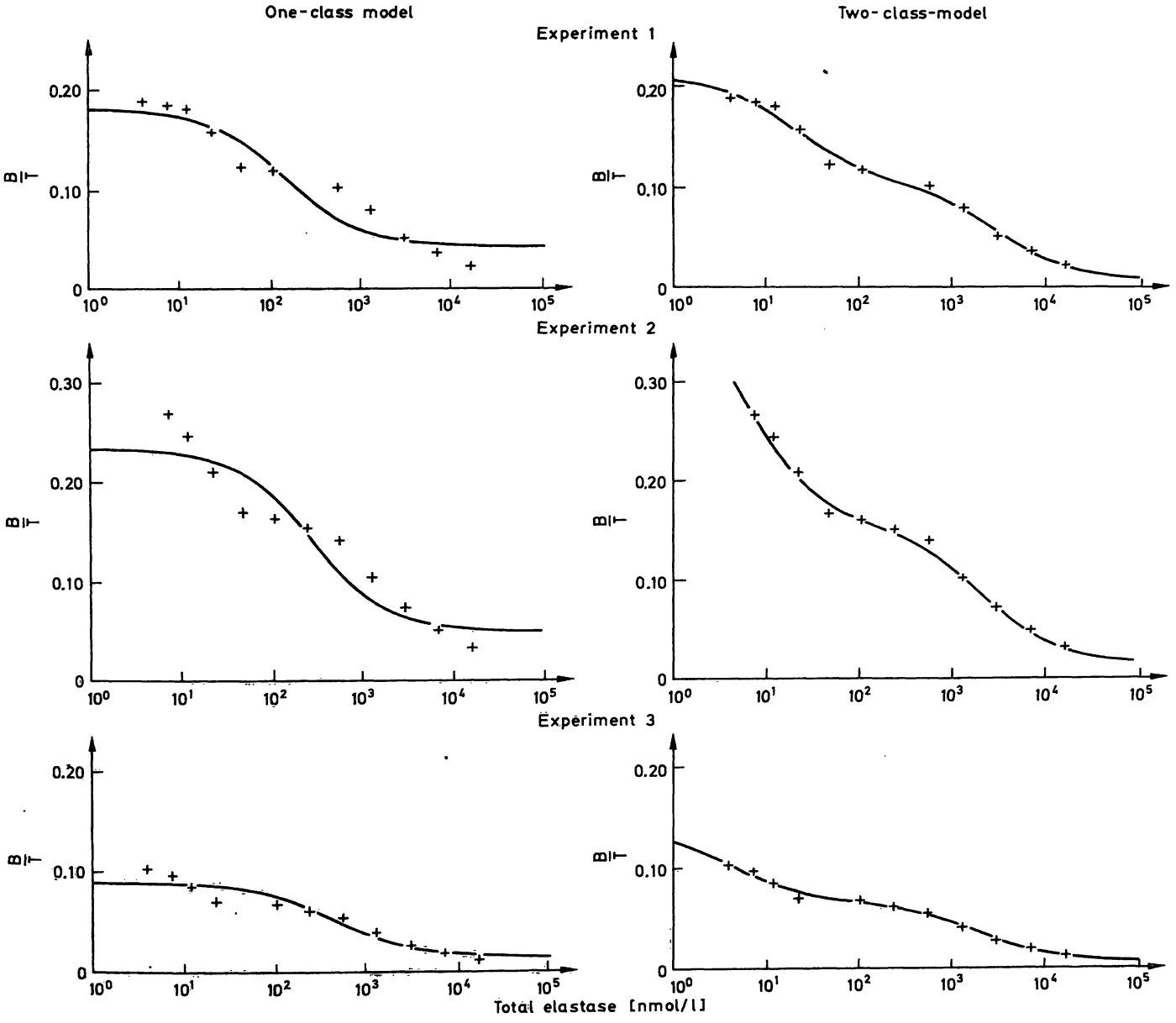

Fig. 8. Competition inhibition curves from non-linear regression analysis for a one-class model (left side) and a two-class model (right side).

plexes or free elastase with secondary complexation are ingested by granulocytes. From the results it is clear that neither resting nor stimulated granulocytes can take up α_1-proteinase inhibitor-elastase/[^{125}I]elastase complexes. These findings are in agreement with *Dolovich* et al. (3) and *Campbell* et al. (2) who described an uptake by macrophages of proteinase-α_2-macroglobulin but not of proteinase-α_1-proteinase inhibitor complexes. Surprisingly, [^{125}I]elastase was bound by granulocytes that commonly secrete this enzyme if stimulated. In comparison with the α_1-proteinase inhibitor-elastase complex, [^{125}I]elastase alone was bound to a considerably higher extent, and this binding was inhibited or depressed in the presence of the respiratory burst stimulators, 4βphorbol 12β-myristate 13α-acetate, *E. coli* endotoxin, and N-formyl-methionyl-leucyl-phenylalanine. This may be explained by release of elastase (initiated by the respiratory burst) from granulocytes into the cell environment and competition with [^{125}I]elastase for binding to granulocytes, thus causing a decrease of cell-bound [^{125}I]elastase. These observations led us to the assumption of specific elastase binding sites on the granulocyte surface, which we were able to demonstrate by association and dissociation experiments as well as competition inhibition experiments. From the dissociation experiments one can conclude that only a small portion of negatively cooperating site-site interactions may exist, if at all, and the data from the *Scatchard* plot could be interpreted according to the speculation of two independently operating elastase receptor populations. To further substantiate this assumption we tried to calculate the binding characteristics from the experimental data without subtraction of non-specific binding by non-linear regression methods. It was observed that a better fit of the experimental data was achieved by employment of the binding model with two independent receptor sites rather than by the use of a one-class model. The data for the low affinity/high capacity as well as for the high affinity/low capacity binding site are in agreement for the two-class non-linear regression procedure and the *Scatchard* calculation.

In conclusion, polymorphonuclear leukocytes possess a specific elastase receptor system on their surface, the biological significance of which remains to be elucidated. Presumably, the elastase receptor can act to bind and inactivate extracellularly released enzyme and/or receptor-bound enzyme can be internalized and then bound to α_1-proteinase inhibitor. Both mechanisms should provide for the elimination of elastase and in that granulocytes can act as protective cells by partially deactivating the injurious elastolytic potential in the cell environment produced by extracellular elastase release in particular pathophysiological circumstances, like highly stimulated phagocytosis.

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